Remarks

Reconsideration of this Application is respectfully requested.

Upon entry of the foregoing amendment, claims 1-4, 6, 8-11 and 16-21 are pending in the application, with claims 1 and 16 being the independent claims. Claims 5, 12, 13 and 15 are sought to be cancelled without prejudice to or disclaimer of the subject matter therein. Claims 1, 6, 8, 9, 11 and 16 are sought to be amended. Claims 17-21 are sought to be added. No new matter is added by way of these amendments. It is respectfully requested that the amendments be entered and considered.

Based on the above amendment and the following remarks, Applicants respectfully request that the Examiner reconsider all outstanding rejections and that they be withdrawn.

I. Support for Amended and New Claims

Support for amended claim 1 can be found throughout the specification, for example, at page 6, lines 19-26 and at page 8, lines 12-15. Support for amended claims 9 and 16 can be found throughout the specification, for example, at page 10, lines 12-14. Claims 6, 8, 9 and 11 have been amended to correct their dependencies.

Support for new claim 17 can be found throughout the specification, for example, at page 10, lines 15-25. Support for new claims 18 and 21 can be found throughout the specification, for example, at Examples 9-11 (pages 22-33). Support for new claims 19 and 20 can be found throughout the specification, for example, at page 10, lines 8-9.

II. Statutory Double Patenting Rejection Under 35 U.S.C. § 101

Claim 16 was rejected under 35 U.S.C. § 101 as claiming the same invention as that of claims 1, 2, 8 and 9 of U.S. Patent No. 6,723,532. *See* Office Action, page 3.

At the outset, Applicants note that in the rejection under § 101 the Examiner has referred to two different patent numbers -- U.S. Patent No. 6,723,938 and U.S. Patent No. 6,723,532. From a review of the two disclosures, it appears that the intended rejection was based on U.S. Patent No. 6,723,532. Also, the Examiner initially indicated that claim 16 is obvious in view of issued claims 1, 2, *8 and 9*. However, the body of the rejection refers to issued claims 1, 2, *9 and 11*. From a review of the two claim sets, it appears that the Examiner intended the rejection to be based on claims 1, 2, 9 and 11. Thus, the intended rejection of claim 16 under 35 U.S.C. § 101 appears to be based on claims 1, 2, 9, and 11 of U.S. Patent No. 6,723,532.

A statutory double patenting rejection cannot be properly made if, for example, there is an embodiment of the invention that falls within the scope of a claim in the application but does not fall within the scope of a corresponding claim in the patent, or *vice versa*. *See In re Vogel*, 422 F.2d 438, 164 USPQ 619 (CCPA 1970); *see also* MPEP § 804, II, A. Claims 1, 2, 9 and 11 of the '532 patent encompass subject matter that does not fall within the scope of claim 16 as currently presented. Claim 16 as currently presented encompasses subject matter that does not fall within the scope of claims 1, 2, 9 and 11 of the '532 patent. Thus, claim 16 of the present application and claims 1, 2, 9 and 11 of the '532 patent do not define the

"same invention" and a statutory double patenting rejection cannot be maintained. Applicants respectfully request that this rejection be reconsidered and withdrawn.

III. Nonstatutory Double Patenting Rejections

The Examiner has made four separate provisional nonstatutory double patenting rejections:

- Claim 16 was provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-3 of copending Application No. 09/728,207. *See* Office Action, page 4;
- Claim 16 was provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-3 of copending Application No. 09/720,003. *See* Office Action, page 5;
- Claims 1-6, 8-11, 15 and 16 were provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-6, 8-10 and 14-18 of copending Application No. 09/720,979. *See* Office Action, page 6; and
- Claim 16 was provisionally rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claim 1 of copending Application No. 10/444,661. *See* Office Action, page 8.

Applicants respectfully request that these rejections be held in abeyance until allowable subject matter is established. At that stage, Applicants will consider filing terminal disclaimer(s) over the above-cited patent applications.

IV. Priority

The Examiner indicated that the application should contain a reference to the earlier filed applications for which priority is claimed. *See* Office Action, page 9. Applicants have inserted at the beginning of the specification a paragraph captioned "Cross-Reference To Related Applications," specifying that the present application is a continuation-in-part of U.S. Patent Application No. 09/720,979, filed March 7, 2001, which is a 371 of International Patent Application No. PCT/JP99/03552, filed on July 1, 1999, which claims the benefit of Japanese Patent Application No. 10/204333, filed on July 3, 1998.

Applicants note that a proper priority claim to the aforementioned applications has been timely made in the present application. *See, e.g.*, Continuation-in-Part Application Transmittal, dated April 30, 2001, and the Combined Declaration and Power of Attorney for Patent Application, submitted on August 30, 2001.

V. Claim Rejections Under 35 U.S.C. § 112, First Paragraph

A. New Matter

Claims 1-6, 8-11, 15 and 16 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. *See* Office Action, page 10. According to the Examiner, the expression "local delivery" in claims 1 and 16 is considered new matter. *See* Office Action, page 11.

Amended claims 1 and 16 do not recite the expression "local delivery." Thus, this rejection has been fully accommodated and should be withdrawn.

B. Written Description

Claims 9 and 10 were rejected under 35 U.S.C. § 112, first paragraph, as allegedly failing to comply with the written description requirement. *See* Office Action, page 11. According to the Examiner, the specification "is not deemed sufficient to reasonably convey to one skilled in the art that Applicant [was] in possession of any neurotrophic factor or any protein that protects the brain from ischemia, at the time the application was filed." *See* Office Action, page 13. Applicants respectfully traverse this rejection.

Claim 9, as currently presented, is directed to the method of claim 1, wherein the foreign gene of the viral vector encodes a protein capable of protecting the brain from ischemia selected from the group consisting of fibroblast growth factors, nerve growth factors, apoptosis inhibitors, heat shock proteins, peroxidases, and neurotrophic factors. Claim 10 depends from claim 9 and specifies that the protein is a neurotrophic factor.

To satisfy the written description requirement of 35 U.S.C. § 112, first paragraph, an applicant must convey with reasonable clarity to those skilled in the art that, as of the effective filing date, the applicant was in possession of the invention. *See Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1560, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). The written description requirement for a claimed genus can be satisfied, *e.g.*, by disclosure of relevant, identifying characteristics, *i.e.*, structure or other physical and/or chemical properties, by functional characteristics coupled with a known or disclosed correlation between function and structure, or by a combination of such identifying characteristics, sufficient to show that

the Applicant was in possession of the claimed genus. *See Regents of the University of California v. Eli Lilly*, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997).

According to the USPTO's guidelines for determining adequacy of written description, the written description requirement for a claimed genus may be satisfied through sufficient description of a representative number of species. *See* M.P.E.P. § 2163. The USPTO's guidelines further note that "[w]hat constitutes a 'representative number' is an inverse function of the skill and knowledge in the art." *See Id.*

The present specification describes several exemplary proteins capable of protecting the brain from ischemia. As discussed below, the level of knowledge and skill in the art relating to the particular categories of proteins recited in claims 9 and 10 is very high. Moreover, the species found within the individual categories of proteins recited in claims 9 and 10 are not substantially variable. Therefore, the exemplary species of proteins capable of protecting the brain from ischemia disclosed in the specification would be regarded as a "representative number" under 35 U.S.C. § 112, first paragraph.

With respect to fibroblast growth factors and nerve growth factors, the specification describes several exemplary species including FGF-1, FGF-2, FGF-5, NGF, CNTF, BDNF, and GDNF, which are capable of protecting the brain from ischemic injury. *See* specification at page 10, lines 15-20. It is well known in the art that fibroblast growth factors (FGFs) and nerve growth factors (NGFs) regulate the survival and growth of neurons in the brain and peripheral tissue and play a role in self-protection against ischemia and, thus, can inhibit post ischemic neuronal death when administered before or immediately after ischemia modalities of cerebral protection. Since the level of knowledge and skill in

the field of FGFs and NGFs is very high, the disclosed species of FGFs and NGFs would be regarded as a representative number under 35 U.S.C. § 112, first paragraph.

With respect to apoptosis inhibitors, heat shock proteins and peroxidases, the specification describes several exemplary species including CrmA, ILP, bcl-2, and ORP-150, which are capable of protecting the brain from ischemic injury. *See* specification at page 10, lines 20-25. It is appreciated in the art, based on clinical results, that apoptosis is a crucial event that can initiate reperfusion-induced inflammation post-ischemia and subsequent tissue injury; thus, apoptosis inhibitors find utility in protecting tissues, including brain tissues, from ischemic damage. Likewise, heat shock proteins and peroxidases have been shown to play a role in protecting tissues from ischemic damage. Since the level of knowledge and skill in the field of apoptosis inhibitors, heat shock proteins and peroxidases, is very high, the disclosed species of apoptosis inhibitors, heat shock proteins and peroxidases would be regarded as a representative number under 35 U.S.C. § 112, first paragraph.

With respect to neurotrophic factors, the specification describes several exemplary species including NGF (nerve growth factor), CNTF (ciliary neurotrophic factor), BDNF (brain-derived neurotrophic factor or neurotrophin-2), and GDNF (glial cell-derived neurotrophic factor). *See* specification at page 10, lines 15-20. The ordinary meaning of the term "neurotrophic" factor is a factor involved in the nutrition and maintenance of neural tissue, for example, a nerve growth factor. See The On-line Medical Dictionary (http://cancerweb.ncl.ac.uk/omd/index.html). Other well known examples of neurotrophic factors include members of the neurotrophin family, which includes, in addition to BDNF, neurotrophin-3, neurotrophin-4, and beta-nerve growth factor. All of these "neurotrophic

factors" are broadly categorized as cystine knot cytokines, a family of small proteins having a disulfide-rich fold and sharing a common core that is all-beta. *See* the SCOP database at http://scop.berkeley.edu/index.html. Thus, the genus of "neurotrophic factors" is a well-studied and well-characterized family of proteins that is not substantially variable. Moreover, given the skill and knowledge in the art, the examples disclosed in the instant specification constitute a representative number of species sufficient to demonstrate possession thereof.

In view of the exemplary species of fibroblast growth factors, nerve growth factors, apoptosis inhibitors, heat shock proteins, peroxidases and neurotrophic factors, and the high level of knowledge and skill in the art regarding these categories of proteins, a person of ordinary skill in the art would recognize that, as of the effective filing date of the present application, Applicants were in possession of the full scope of subject matter encompassed by currently presented claims 9 and 10. Applicants respectfully request that the written description rejection be reconsidered and withdrawn.

C. Enablement

Claims 1-6, 8-11, 15 and 16 were rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement. *See* Office Action, page 13. According to the Examiner, the specification:

> does not reasonably provide enablement for any method of administration, any viral vector, any ex vivo method, transgenes located in any part of the viral genome, or the treatment of any mammal. The specification does not enable

> any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention commensurate in scope with these claims.

See Office Action, page 14. Applicants respectfully traverse this rejection.

In order to establish a *prima facie* case of lack of enablement, the Examiner has the initial burden to set forth a reasonable basis to question the enablement provided for the claimed invention. *See In re Wright*, 999 F.2d 1557, 1562, 27 USPQ2d 1510, 1513 (Fed. Cir. 1993). To satisfy this burden, "it is incumbent upon the Patent Office. . . to explain *why* it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." *See In re Marzocchi*, 439 F.2d 220, 224, 169 USPQ 367, 370 (CCPA 1971) (emphasis in original). As explained below, the Examiner has not established that it would have required undue experimentation on the part of one of ordinary skill in the art to practice the methods encompassed by the currently presented claims.

As an initial matter, Applicants note that claims 1 and 16 have been amended to specify that the vector is a Sendai viral vector comprising a foreign gene, and that the foreign gene is inserted between the R1 and R2 loci of the Sendai virus. Thus, the enablement rejection, insofar as it relates to the nature of the viral vector and the location of the foreign gene in the vector, has been fully accommodated.

The Examiner has cited several references to support the assertion that making and using the subject matter of the present invention would have required undue experimentation. Applicants submit that the documents cited by the Examiner either do not reflect the state of the art at the time the present application was filed, are not pertinent to the

claimed invention, or both. Specifically, the cited references fail to challenge the asserted utility and operability of Sendai viral vectors.

Of the cited references, only Yonemitsu and Nakanishi specifically mention Sendai viruses. Applicants submit that, contrary to the Examiner's suggestion, neither reference undermines the enablement of Applicants' claimed invention. Regarding Nakanishi, it is unclear how his findings support the premise that one skilled in that art could not reasonably predict that Sendai viral vectors act similarly to Nakanishi's fusogenic liposomes in terms of vector targeting. *See* Office Action, page 21. The fusogenic liposomes (FL) described by Nakanishi comprise a fusion of simple liposomes and Sendai virus particles, notably the SeV envelope protein. As simple liposomes lack any targeting mechanism, clearly any positive targeting that occurs is the result of the inclusion of the SeV envelope proteins which are responsible for delivering the FL to the cytosol. Furthermore, more recent publications of Nakanishi et al. emphasize the advantages of the fusogenic liposomes, noting that the FL is an efficient tool for the delivery of CTL vaccines (see Eur J Immunol. 2000 Jun;30(6):1740-7, abstract attached hereto as Exhibit 1), a versatile and effective system for the stimulation of Ag-specific immune responses at both mucosal and systemic compartments (see J Immunol. 2001 Aug 1;167(3):1406-12, copy attached hereto as Exhibit 2) and a potentially a useful delivery vehicle for oligonucleotide-based therapeutics (see Biol Pharm Bull. 2000 Aug;23(8):1011-3, abstract attached hereto as Exhibit 3). Accordingly, contrary to the Examiner's suggestion, Nakanishi et al. support the operability and utility of Sendai viral vectors to deliver exogenous genes to specific tissues, such as nerve tissues.

Regarding Yonemitsu, the majority of concerns mentioned specifically relate to the practicalities of gene therapy for cardiovascular disorders, noting the complexity of the disease, the wide range of factors involved, and lack of pathophysiologic understanding of underlying causes. None of these concerns specifically apply to or challenge the utility of Sendai viral vectors to deliver therapeutic genes to nerve tissues, particularly in light of Applicants' documented *in vitro* and *in vivo* successes. *See* Examples 6-11 of the present specification. Furthermore, like Nakanishi, Yonemitsu in fact supports the enablement of Applicants' invention. Specifically, in his optimistic conclusion, he notes that the newly developed recombinant Sendai virus shows dramatically superior gene-transfer efficiency to other vectors, including adenovirus, in several organs and therefore offers new possibilities in the filed of gene therapy. Thus, one reading Yonemitsu would have no cause to doubt the enablement of the present invention.

The remainder of the cited references, particularly Verma, discuss drawbacks associated with viral vectors derived from retroviruses, lentiviruses, and adenoviruses, as well as other viruses such as HSV, HIV, HTLV, MuLV, Sindbis virus, semliki forest virus, and vaccinia virus. However, none of these are negative strand RNA viruses, much less Paramyxoviruses or Sendai viral vectors. Because negative strand RNA viruses are not integrated into the chromosomes, they are expected to be safer. They are also expected to have reduced cytotoxicity and immunogenicity. Furthermore, RNA virus have advantages, such as "protein expression in short time after infection and an extremely higher level expression of the transgene product compared with adenovirus." *See* specification, page 2, lines 4-7. Thus, references discussing the hurdles associated with adenoviral vectors and the

like are not dispositive on the issue on the enablement of Paramyxoviral vectors, more particularly Sendai viral vectors. Moreover, Verma's generic doubts of the prior art, circa 1997, are assuaged by the concrete proof of principle experiments set forth in Applicants' specification, circa 2001. See particularly Examples 6-11 which conclusively demonstrate the ability of the Sendai viral vector not only to deliver a desired foreign gene to a target cell population (Examples 6 and 7) but to also provide levels of protein sufficient to confer therapeutic benefit (Examples8-11). Finally, while Verma *et al.* note that the inability to deliver genes efficiently and to obtain sustained expression are indeed major problems, they conclude that these problems are surmountable and that in the not too distant future, gene therapy will become as routine a practice as heart transplants are today. Thus, gene therapy is not *per se* a suspect science. Moreover, the Verma reference was published in1997. Clearly numerous advances had been made in the field by the time the present application was filed.

The Examiner cites to Eck and Deonarian in support of the premise that tissue targeting and long term expression are major problems in the field of gene therapy. However, their generic warnings, circa 1996 and 1998, respectively, are outweighed by Applicants specifically documented successes. *See* discussion above. In the instant specification, Applicants have objectively and conclusively demonstrated that *in vivo* intraventricular delivery of a foreign-gene-carrying recombinant Sendai viral vector to a number of different animal systems (e.g., mice and gerbils) results in dose-dependent expression of functional protein in specific nerve cells and nervous tissues, at levels far superior to those associated with prior art vector systems, such as adenoviral vectors.

Accordingly, Applicants have already demonstrated that tissue targeting and protein expression are not a significant hurdles to *in vivo* applications of the instant invention.

The Examiner cites to Gorecki *et al.* for the premise that one must specifically tailor the delivery system to the individual disease. This is exactly what Applicants have done. They have demonstrated that the specifically claimed vector (i.e., Paramyxoviral vectors) can deliver nucleic acids to specific cells (i.e., nerve cells such as ependymal cells and pyramidal cells of the hippocampus) *in vivo* with the requisite level of efficiency necessary to ensure therapeutic results (again, see Examples 8-11).

The Examiner cites to Crystal and Gura in support of the premise that gene therapy cannot be extrapolated from animal models to human systems. At the outset, it is important to note that neither Crystal nor Gura, represent the state of the art at the time of the instant invention. Many advances had been made in the field of gene therapy in the intervening years. Moreover, Applicants believe the Examiner has taken various statements from these references out of context. In the opening abstract, Crystal expressly states that "enough information has been gained from clinical trials to allow the conclusion that human gene transfer is feasible, can evoke biologic responses that are relevant to human disease" and that "adverse events have been uncommon and have been related to the gene delivery strategies (i.e., the delivery system);" while "human gene transfer still faces significant hurdles before it becomes an established therapeutic strategy", "its accomplishments to date are impressive and the logic of the potential usefulness of the clinical paradigm continues to be compelling".

Finally, Applicants respectfully remind the Examiner that the proper standard for compliance with the enablement requirement is not absolute predictability but objective enablement; *i.e.*, would one reasonably skilled in the art be able to make and use the claimed invention from the disclosures in the patent coupled with information known in the art without undue experimentation? Evidence provided by Applicants need not be conclusive but merely convincing to one of skill in the art (see MPEP § 2164.05). In this case, Applicants submit that the compelling animal data presented in the specification is sufficiently "convincing" that one of ordinary skill in the art would not doubt its feasibility or its application to mammals other than rodents. Moreover, the *in vivo* successes documented in the Examples of the instant specification clearly outweigh the prior art's speculative allegations of unpredictability.

In the instant case, the positive findings associated with the *in vitro* and *in vivo* delivery and expression in specific nerve cells of both reporter genes, such as firefly luciferase and β-galactosidase, and therapeutic genes, such as β-glucuronidase, directly correlate to treatment of gene-based diseases in animals, including humans. Given this correlation, there is no reason to doubt Applicants' assertion that the Sendai viral vectors of the present invention would be useful in treating neurodegenerative conditions, such as those associated with Parkinson's disease, ischemia and the like.

Thus, for the reasons given above, Applicants submit that a person of ordinary skill in the art would have been able to make and use the full scope of the subject matter encompassed by the present claims. Applicants respectfully request that the enablement rejections be reconsidered and withdrawn.

VI. Claim Rejections Under 35 U.S.C. § 102

A. Hasan

Claim 16 was rejected under 35 U.S.C. § 102(b) as being anticipated by Hasan *et al.*, *J. Gen. Virol. 78*:2813-2820 (1997) ("Hasan"). *See* Office Action, page 25. Applicants respectfully traverse this rejection.

An anticipation rejection under 35 U.S.C. § 102 requires a showing that each limitation of a claim is found in a single reference, practice, or device. *See In re Donohue*, 766 F.2d 531, 226 USPQ 619, 621 (Fed. Cir. 1985). Claim 16, as currently presented, is directed to a Sendai viral vector comprising a Sendai viral genome and a foreign gene. The foreign gene is specified to encode a protein capable of protecting the brain from ischemia. Hasan does not teach a Sendai viral vector comprising a foreign gene that encodes a protein capable of protecting the brain from ischemia. Therefore, Hasan does not and cannot anticipate claim 16. Applicants respectfully request that this rejection be reconsidered and withdrawn.

B. Patent Application Publication No. US 2002/0100066

Claim 16 was rejected under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent Application Publication No. 2002/0100066, filed September 13, 2001, published July 25, 2002, and claiming priority to Japanese application number 7/308315, filed October 31, 1995. *See* Office Action, page 26. Applicants respectfully traverse this rejection.

U.S. Patent Application Publication No. 2002/0100066 does not teach a Sendai viral vector comprising a foreign gene that encodes a protein capable of protecting the brain from ischemia. Therefore, U.S. Patent Application Publication No. 2002/0100066 does not and cannot anticipate claim 16. Applicants respectfully request that this rejection be reconsidered and withdrawn.

C. Patent Application Publication No. US 2002/0098576

Claim 16 was rejected under 35 U.S.C. § 102(e) as being anticipated by U.S. Patent Application Publication No. 2002/0098576, filed December 1, 2000, published July 25, 2002, and claiming priority to Japanese application number 7/285417, filed November 1, 1995. *See* Office Action, page 27. Applicants respectfully traverse this rejection.

U.S. Patent Application Publication No. 2002/0098576 does not teach a Sendai viral vector comprising a foreign gene that encodes a protein capable of protecting the brain from ischemia. Therefore, U.S. Patent Application Publication No. 2002/0098576 does not and cannot anticipate claim 16. Applicants respectfully request that this rejection be reconsidered and withdrawn.

Conclusion

All of the stated grounds of rejection have been properly traversed, accommodated, or rendered moot. Applicants therefore respectfully request that the Examiner reconsider all presently outstanding rejections and that they be withdrawn. Applicants believe that a full and complete reply has been made to the outstanding Office Action and, as such, the present application is in condition for allowance. If the Examiner believes, for any reason, that personal communication will expedite prosecution of this application, the Examiner is invited to telephone the undersigned at the number provided.

Prompt and favorable consideration of this Amendment and Reply is respectfully requested.

Respectfully submitted,

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1: Eur J Immunol. 2000 Jun;30(6):1740-7.

Fusogenic liposomes efficiently deliver exogenous antigen through the cytoplasm into the MHC class I processing pathway.

Nakanishi T, Hayashi A, Kunisawa J, Tsutsumi Y, Tanaka K, Yashiro-Ohtani Y, Nakanishi M, Fujiwara H, Hamaoka T, Mayumi T.

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Exogenous soluble proteins enter the endosomal pathway by endocytosis and are presented in association with MHC class II rather than class I. In contrast, the delivery of exogenous protein antigens (Ag) into the cytosol generates MHC class I-restricted cytotoxic T lymphocytes (CTL) responses. Although several immunization approaches, such as the utilization of liposomes, have induced the in vivo priming of MHC class I-restricted CTL responses to protein Ag, it remains unclear whether this priming results from the direct delivery of protein Ag to the cytosol. Here we report that fusogenic liposomes (FL), which are prepared by fusing simple liposomes with Sendai virus particles, can deliver the encapsulated soluble protein directly into the cytosol of cells cultured concurrently and introduce it into the conventional MHC class I Ag presentation pathway. Moreover, a single immunization with ovalbumin (OVA) encapsulated in FL but not in simple liposomes results in the potent priming of OVA-specific CTL. Thus, FL function as an efficient tool for the delivery of CTL vaccines.

PMID: 10898512 [PubMed - indexed for MEDLINE]

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Sendai Virus Fusion Protein-Mediates Simultaneous Induction of MHC Class I/II-Dependent Mucosal and Systemic Immune Responses Via the Nasopharyngeal-Associated Lymphoreticular Tissue Immune System[1]

Jun Kunisawa,*† Tsuyoshi Nakanishi,* Ichiro Takahashi,† Akiko Okudaira,*† Yasuo Tsutsumi,* Kazufumi Katayama,*† Shinsaku Nakagawa,* Hiroshi Kiyono,† and Tadanori Mayumi[2]*

Nasal administration of Ags using a novel hybrid Ag delivery vehicle composed of envelope glycoproteins of Sendai virus on the surface of liposome membranes (fusogenic liposome) efficiently delivered Ags to Ag-sampling M cells in nasopharyngeal-associated lymphoreticular tissue. Additionally, fusogenic liposomes also effectively delivered the Ags into epithelial cells and macrophages in nasopharyngeal-associated lymphoreticular tissue and nasal passages. In vitro Ag presentation assays clearly showed that fusogenic liposomes effectively presented encapsulated Ags via the MHC class II-dependent pathway of epithelial cells as well as macrophages. Fusogenic liposomes also have an adjuvant activity against mucosal epithelial cells to enhance MHC class II expression. According to these high delivery and adjuvant activities of fusogenic liposomes, nasal immunization with OVA-encapsulated fusogenic liposomes induced high levels of OVA-specific $CD4^+$ Th1 and Th2 cell responses. Furthermore, Ag-specific CTL responses and Ab productions were also elicited at both mucosal and systemic sites by nasal immunization with Ag-encapsulated fusogenic liposomes. These results indicate that fusogenic liposome is a versatile and effective system for the stimulation of Ag-specific immune responses at both mucosal and systemic compartments. *The Journal of Immunology,* **2001, 167: 1406–1412.**

The mucosal surfaces are primary sites that many virus and bacteria invade to establish infection. To prevent such microorganism transmission across the mucosal epithelium and dissemination to the regional lymph nodes or target organ, much effort has been focused on the development of a mucosal vaccine because oral or nasal immunization elicits both mucosal and systemic immune responses (1–3). Such responses include secretory IgA and IgG Abs that are reported to mediate microorganism neutralization and prevent adhesion to the epithelium. In addition, induction of CTL responses may be essential for virus clearance from mucosal tissue to prevent virus dissemination if the mucosal barrier is destroyed (4, 5).

It has been shown that mucosal delivery of soluble Ag alone is insufficient for the induction of sufficient levels of Ag-specific immune responses. In this respect, various attempts have aimed to improve the efficacy of mucosal vaccines using mucosal adjuvant (6–8) or Ag delivery systems (9, 10). We have developed fusogenic liposomes, consisting of liposomes fused with Sendai virus, attached and fused cells, and delivered their encapsulated protein and plasmid DNA into the cytoplasm of the attached cells (11–13). We also reported that s.c. immunization with Ag-encapsulated fusogenic liposomes induced Ag-specific CTL responses at systemic lymphoid tissues in a MHC class I-dependent manner (14) in addition to Ag-specific Ab production in sera (15). Because Sendai virus naturally infects via the mucosal epithelium (16, 17), fusogenic liposomes may effectively deliver the Ag to the mucosal immune system and induce Ag-specific mucosal and systemic immune responses.

The present study demonstrates the effectiveness of fusogenic liposomes as a new and novel nasal vaccine vehicle with which to generate optimal nasopharyngeal-associated lymphoreticular tissue (NALT)[3]-mediated mucosal as well as systemic immune responses.

Materials and Methods

Mice and cell lines

Female C57BL/6 and BALB/c mice (7–10 wk old) were purchased from Charles River Breeding Laboratories (Yokohama, Japan). The $CD4^+$ T cell hybridoma, 3A9, specific for peptide 46–61 of hen egg white lysozyme (HEL) in the context of $I\text{-}A^k$, was provided by Dr. P. Allen (Washington University, St. Louis, MO) (18). MODE-K, an intestinal epithelial cell line, was provided by Dr. D. Kaiserlian (Institute Pasteur, Lyon, France) (19). IL-2-dependent CTLL-2 cell was a kind gift from Dr. T. Hamaoka (Osaka University, Osaka, Japan). EL4, a C57BL/6 mice-derived T lymphoma, was obtained from Tohoku University (Sendai, Japan). EG7, which is a

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[3] Abbreviations used in this paper: NALT, nasopharyngeal-associated lymphoreticular tissue; HEL, hen egg white lysozyme; UEA-1, ulex europaeus agglutinin-1; CLN, cervical lymph nodes; MLN, mesenteric lymph nodes; PBS-T, PBS containing 0.05% Tween 20; AFC, Ab-forming cell.

0022-1767/01/$02.00

chicken egg OVA gene-transfected clone of EL4 and which presents OVA with MHC class I molecules, was obtained from the American Type Culture Collection (Manassas, VA) (20).

Preparation of fusogenic liposomes

Fusogenic liposomes were prepared as described previously (14, 15, 21, 22). Briefly, lipid mixture (L-α-dimyristoyl phosphatidic acid/phosphatidylcholine/cholesterol in a molar ratio of 1:4:5) was dissolved in chloroform (Sigma, St. Louis, MO). The solvent was subsequently evaporated to obtain a thin lipid film. Conventional liposomes were prepared by dispersing the thin lipid film with a given amount of FITC-labeled dextran (50 mg/ml), OVA (100 mg/ml), or HEL (50 mg/ml) solution using a vortex and freeze-thaw method, and sized by extrusion through a 400-nm polycarbonate membrane. Sized liposomes were mixed with UV-inactivated Sendai virus and incubated at 37°C for 2 h with shaking. Fusogenic liposomes were finally purified by sucrose step centrifugation (24,000 rpm, 2 h, 4°C).

In vitro uptake analysis using confocal microscopy

MODE-K cells were cultured overnight. After removing the supernatant, various FITC-dextrans (100 μg/ml) were added, and the cells were cultured for 1 h at 37°C, washed, and observed using a confocal microscope (Bio-Rad, Hercules, CA).

Ag presentation assay

Ag presentation was assayed as described (19). Briefly, macrophages isolated from peritoneal exude cells or IFN-γ-treated (50 U/ml for 3 days) MODE-K cells were cultured with 50 μg/ml mitomycin C at 37°C for 45 min. The cells were washed and incubated for 2 h at 37°C (5×10^4 cells/well). Various concentrations of HEL (0.1, 1, 10, 100 μg/ml) or OVA (100 μg/ml) in fusogenic liposomes were added and cocultured for an additional 5 h. After incubation, the cells were washed, and HEL-specific T-T hybridomas (3A9 cells) were added (5×10^4 cells/well). After 24 h of culture, the supernatants were collected, and IL-2 production in the supernatants was quantified using an IL-2-dependent CTLL-2 cell line.

Analysis of MHC class II expression on epithelial cells

To determine whether fusogenic liposomes enhance MHC class II expression of epithelial cells, MODE-K cells were cultured with the same lipid concentration of fusogenic liposomes, conventional liposomes, or Sendai virus for 48 h. Following three washings, cells were incubated with anti-CD16/32 (Fc block; BD PharMingen, San Diego, CA) for 15 min at room temperature and then stained with PE-labeled anti-I-A^k Ab (BD PharMingen) for 30 min at 4°C. These cells were washed three times and analyzed by flow cytometry analysis using a FACScan flow cytometer (Becton Dickinson, Mansfield, MA).

Isolation of mononuclear cells

Mononuclear cells from the nasal passages, NALT, mesenteric lymph nodes (MLN), intestinal lamina propria, cervical lymph nodes (CLN), and spleen were isolated as previously described (7, 8, 23). In brief, mononuclear cells from NALT and nasal passages were prepared as follows. Pear-shaped NALT was removed from the palate. After the removal of NALT, the nasal passages were also isolated from the nasal cavity. Mononuclear cells from the CLN, MLN, and spleen were also isolated using mechanical dissociation. Intestinal lamina propria mononuclear cells were isolated by an enzymatic dissociation procedure with collagenase type IV (Sigma).

In vivo Ag distribution assay

Mice were nasally administered with various formed FITC-dextrans (5 mg/ml; Sigma). After 1 h, mononuclear cells were isolated from NALT and nasal passages as described above, then epithelial cells were purified by discontinuous Percoll gradient centrifugation (25 and 40%) according to the method described previously (24). Mac-1^+ cells were detected using a PE-labeled anti-Mac-1 Ab (Caltag, Burlingame, CA). Fluorescence-positive cells were measured using a FACScan flow cytometer.

Immunohistological analysis using confocal microscopy

M cells were detected using whole-mount staining with the M cell-specific lectin, ulex europaeus agglutinin-1 (UEA-1; Vector Laboratories, Burlingame, CA) (25, 26). One hour after nasal administration with fusogenic liposomes containing FITC-dextrans, palates were dissected and fixed with 4% paraformaldehyde at 4°C for 4 h. The specimens were then blocked with diluted (2×) Block Ace (Dai-Nippon Pharmaceutical, Osaka, Japan) for 1 h at room temperature and then stained with PBS containing rhodamine-labeled UEA-1 (20 μg/ml) for 2 h at room temperature. The specimens were finally washed and examined by a confocal microscope.

Immunization

Mice were nasally immunized with 10-μl aliquots of fusogenic liposomes or conventional liposomes containing 50 μg of OVA on days 0, 7, and 14. Another group of mice was nasally immunized with OVA alone.

Proliferative responses of Ag-specific $CD4^+$ T cells

Seven days after the final immunization, lymphocytes were obtained from spleen, CLN, NALT, nasal passages, and MLN. $CD4^+$ T cells were then purified by using anti-mouse CD4 (L3T4)-coupled microbeads and MACS column (Miltenyi Biotec, Sunnyvale, CA) (8). Purified $CD4^+$ T cells were cultured at a density of 2×10^6 cells/ml with 1 mg/ml OVA in the presence of irradiated (3000 rad) splenic feeder cells (2×10^6 cells/ml) at 37°C for 96 h. To measure cell proliferation, 1 μCi of [^{3}H]thymidine was added to individual culture wells 8 h before termination, and the uptake of [^{3}H]thymidine by dividing cells was determined by scintillation counting.

Cytokine analysis by ELISA

Cytokine levels in culture supernatants of Ag-stimulated $CD4^+$ T cells were determined by a cytokine-specific ELISA (7, 8). Briefly, $CD4^+$ T cells obtained from spleen, CLN, NALT, nasal passages, and MLN of the immunized mice were cultured with 1 mg/ml OVA in the presence of irradiated (3000 rad) splenic feeder cells. Culture supernatants were harvested 96 h after incubation, and the levels of Th1 (IFN-γ)- and Th2 (IL-4, IL-5, and IL-6)-type cytokines were determined by cytokine-specific ELISA kit (Amersham Pharmacia Biotech, Piscataway, NJ). The concentration of cytokines was calculated by the standard curves obtained according to the instruction provided by the manufacturer.

Detection of OVA-specific Ab production by ELISA

A standard isotype and Ag-specific ELISA was used in this study (6–9). ELISA plates were coated with 10 μg/ml OVA in 50 mM bicarbonate buffer. Wells were blocked with 2-fold diluted Block Ace (Dai-Nippon Pharmaceutical) for 1 h at room temperature. After washing four times with PBS containing 0.05% Tween 20 (PBS-T), each diluted serum, nasal washes and fecal extracts were added in duplicate (50 μl/well). Serum and fecal extracts from nonimmunized mice were included as controls. Biotin-labeled anti-mouse IgG, IgG1, IgG2a, IgG2b, IgG3, IgM, or IgA (Southern Biotechnology Associates, Birmingham, AL) were used as the detection Ab. Following 8 h incubation at room temperature, plates were washed, and HRP-conjugated strepavidin (Life Technologies, Gaithersburg, MD) was added. The reaction was developed by 3,3′,5,5′-tetramethylbenzidine (Moss, Pasadena, CA), and color development was terminated after a 15-min incubation by addition of 0.5 N HCl. Endpoint titers were expressed as the reciprocal $\log_2$ of the last dilution, which gave an OD at 450 nm of 0.1 greater than nonimmunized mice.

Detection of OVA-specific Ab-forming cells (AFCs)

Seven days after the final immunization, mononuclear cells were obtained from spleen, nasal passages, and intestinal lamina propria. To assess the numbers of OVA-specific AFCs, an ELISPOT assay was used (6–8). Briefly, 96-well nitrocellulose plates (Millititer; Millipore, Bedford, MA) were coated with OVA (1 mg/ml in PBS) and blocked with RPMI 1640 containing 10% FCS. The blocking solution was discarded, and 100 μl of cells in complete RPMI 1640 at various dilutions was added. Following 5 h incubation at 37°C, plates were washed three times with PBS and PBS-T. The detection Ab for IgM, IgG, and IgA isotypes conjugated with HRP (Southern Biotechnology Associates) in PBS-T was then added. Following overnight incubation, plates were washed four times with PBS and developed by the addition of 100 μl of 3-amino-9-ethylcarbazole dissolved in 0.1 M sodium acetate buffer containing 0.015% H_2O_2 (Moss) to each well. Plates were incubated at room temperature for 20–30 min and washed with water, and AFCs were determined by direct counting of spots with the aid of a stereomicroscope.

OVA-specific CTL assay

Seven days after the final immunization, a standard CTL assay was performed (14, 27, 28). Briefly, in vivo-primed single cells usually were cultured with mitomycin C-treated (50 μg/ml) EG7 for 5 days to expand Ag-specific CTLs and were used as effector cells. EL4 and EG7 were labeled with ^{51}Cr for 60 min and added to serially diluted effector cells in 96-well microplates. After a 4-h incubation, ^{51}Cr levels in the supernatants were determined using a gamma counter. The specific lysis of target cells was determined as follows: (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm) × 100.

RT-PCR

Seven days after the final immunization, total RNA was isolated from nasal passage lymphocytes (8). Complementary DNA was synthesized using the standard method and amplified by PCR (8). After 30 cycles of amplification using specific primers (sense for perforin, GGAATTCAGATCGGAG GATTTTAAA; antisense for perforin, GACTACTGTGCCTGCAG CATC) (29), the amplified products were separated by electrophoresis in 1.8% agarose gel and visualized with ethidium bromide.

Statistics

The results were compared using Student's t test and Welch's t test. The values were considered statistically significant when $p < 0.05$.

Results

Fusogenic liposomes effectively deliver the Ag to NALT

To demonstrate the effectiveness of fusogenic liposomes as a mucosal Ag delivery vehicle, mice were nasally administered with FITC-dextran encapsulated in either fusogenic liposomes (FITC-fusogenic liposomes), conventional liposomes (FITC-liposomes), or with FITC alone. One hour later, the fluorescent intensity of epithelial and mononuclear cells isolated from NALT and nasal passages were examined using a FACScan flow cytometer. Both epithelial and Mac-1^+ cells isolated from NALT or nasal passages emitted intense fluorescence following nasal administration with FITC-fusogenic liposomes. In contrast, no cells were fluorescent in either the NALT or nasal passages of mice nasally administered with FITC alone or with FITC-liposomes (Fig. 1, *A–D*). This finding was also supported by an in vitro study showing intense fluorescence in a mucosal epithelial cell line (MODE-K cells) cocultured with FITC-fusogenic liposomes but not with FITC-liposomes or with FITC alone (Fig. 1, *E–H*).

It has been reported that M cells can be recognized by α-L-fucose-specific lectin (UEA-1) in the epithelial layer of NALT (25, 26). These cells are morphologically different from neighboring epithelial cells and specialized for the uptake and transcellular transport of Ags to NALT dendritic cells and macrophages (30–32). In this regard, we examined whether or not fusogenic liposomes have the ability to fuse with M cells for efficient delivery of Ag into NALT. Confocal microscopic analysis using rhodamine-labeled UEA-1 revealed that fusogenic liposomes delivered their contents (FITC-dextran) to M cells as well as to neighboring epithelial cells (Fig. 2).

Fusogenic liposomes can induce MHC class II-restricted Ag presentation and MHC class II expression

Because fusogenic liposomes effectively delivered Ags to mucosal epithelial and Mac-1^+ cells, we examined whether the contents of fusogenic liposomes are presented with MHC molecules. MODE-K cells and freshly isolated macrophages were exposed to fusogenic liposomes containing HEL in vitro. The MHC class II-restricted Ag presentations were briskly noted in macrophages cultured with fusogenic liposomes containing HEL (Fig. 3*A*). Furthermore, the Ag presentation ability of IFN-γ-pretreated MODE-K cells cocultured with fusogenic liposomes containing HEL was also similar (Fig. 3*B*). In contrast, macrophages and MODE-K cells treated with fusogenic liposomes containing irrelevant OVA did not stimulate HEL-specific T cell hybridoma to synthesize IL-2.

It was also shown that several viruses stimulated the expression of MHC molecules and the production of cytokines and chemokines by infected epithelial cells (33, 34). In this respect, we found that fusogenic liposomes as well as Sendai virus activated infected epithelial cells to express MHC class II molecules (Fig. 3*C*). However, conventional liposomes did not affect the MHC class II expression on epithelial cells (Fig. 3*C*).

Fusogenic liposomes induce both Th1- and Th2-type responses

Nasal immunization of Ag with a mucosal adjuvant (e.g., cholera toxin and heat-labile toxin) usually evokes Ag-specific immune responses including Th1- and Th2-type CD4$^+$ T cells in both mucosal and systemic compartments (6–8). In this context, we examined the proliferative response of CD4$^+$ T cells from the NALT, nasal passages, spleen, CLN, and MLN of mice nasally immunized with OVA incorporated into fusogenic liposomes (OVA-fusogenic liposomes) against soluble OVA. As shown in Fig. 4, high levels of OVA-specific proliferative responses were detected in CD4$^+$ T cells isolated from both mucosal (NALT, nasal passages, CLN, MLN) and systemic (spleen) compartments of mice nasally immunized with OVA-fusogenic liposomes. However, OVA-specific proliferative responses were virtually undetectable in mice nasally immunized with OVA using conventional liposomes (OVA-liposomes) or OVA alone (Fig. 4). This finding demonstrated that nasally administered fusogenic liposomes delivered Ag to and stimulated NALT and associated immune compartments.

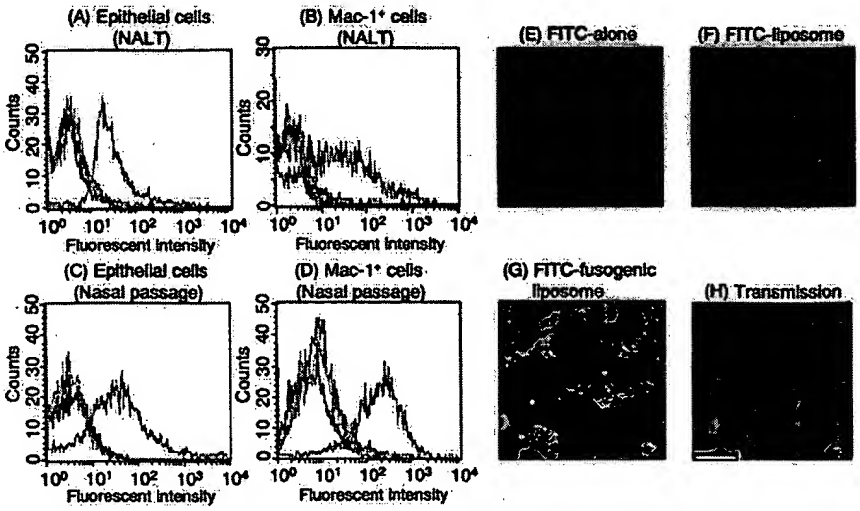

FIGURE 1. In vivo Ag distribution following nasal administration with fusogenic liposomes (*A–D*). One hour after nasal administration with FITC-fusogenic liposomes (red), FITC-liposomes (green), FITC alone (blue), or none (black), epithelial cells (*A*) and Mac-1^+ cells (*B*) of NALT, and epithelial cells (*C*) and Mac-1^+ cells (*D*) of nasal passages were isolated. Fluorescent intensity was detected using a FACScan flow cytometer. Analysis of in vitro Ag uptake by epithelial cells (*E–H*). MODE-K cells were cultured with FITC alone (*E*), FITC-liposomes (*F*), or FITC-fusogenic liposomes (*G*). After 1 h, cells were washed and observed by confocal microscopy. The same sample was observed by transmission microscopy (*H*, white bar indicates 50 μm). The results were confirmed by three independent experiments.

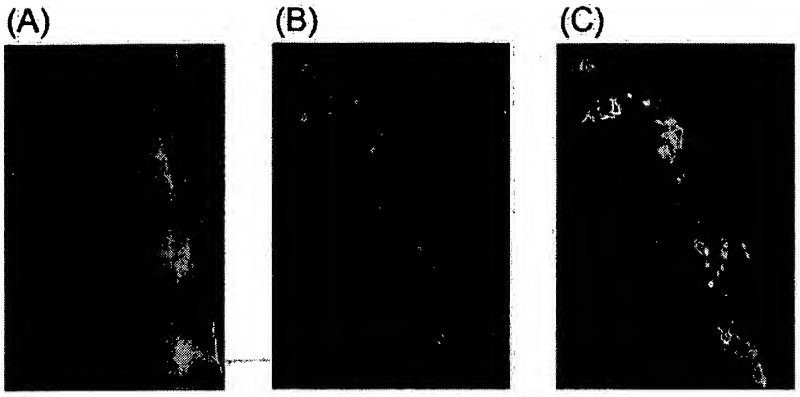

FIGURE 2. Whole mount staining of NALT from mice nasally administered with FITC-fusogenic liposomes using rhodamine-labeled UEA-1. NALT was stained with rhodamine-labeled UEA-1 (*B*). Following nasal administration with FITC-fusogenic liposomes, NALT was then counter stained with rhodamine-labeled UEA-1 (*C*). Yellow and green colors indicate M cells and epithelial cells taken up by Ag, respectively. Findings from three independent experiments were similar.

To characterize Ag-specific $CD4^+$ T cells in the mucosal and systemic tissues of mice nasally immunized with OVA-fusogenic liposomes, Th1 (IFN-γ)- and Th2 (IL-4, IL-5, and IL-6)-specific cytokines production was examined. A large amount of Th1- and Th2-type cytokines were noted in the NALT, nasal passages, CLN, MLN, and spleen of mice immunized with OVA-fusogenic liposomes (Fig. 5). These findings suggest that fusogenic liposomes can induce both mucosal and systemic Th1- and Th2-type $CD4^+$ T cells without a coadministered mucosal adjuvant (e.g., cholera toxin and heat-labile toxin).

Fusogenic liposomes induce Ag-specific humoral immune responses

We next investigated Ag-specific humoral responses at both mucosal and systemic sites. The OVA-specific IgG responses in the serum of mice nasally immunized with OVA-fusogenic liposomes were significantly higher than those of sera from mice immunized with OVA alone or OVA-liposomes (Fig. 6*A*). Consistent with the outcome of the cytokine profile (e.g., IL-4 and IFN-γ) of OVA-specific $CD4^+$ T cells (Fig. 5), high titers of OVA-specific IgG1, IgG2a, and IgG2b were produced in sera (Fig. 6*B*). OVA-specific IgA responses were higher in nasal washes from mice nasally immunized with OVA-fusogenic liposomes than from those immunized with OVA alone or OVA-liposomes (Fig. 6*C*). Fusogenic liposomes also induced OVA-specific IgA responses in fecal extracts (Fig. 6*D*). Results obtained from analyzing Ag-specific AFCs supported the finding of OVA-specific Ab responses in mucosal secretions and serum. Thus, the numbers of OVA-specific IgA and IgG AFCs were increased in the nasal passages, intestinal lamina propria, and spleen of mice nasally immunized with OVA-fusogenic liposomes (Fig. 7). These findings further emphasize the value of fusogenic liposomes as a novel mucosal Ag delivery vehicle.

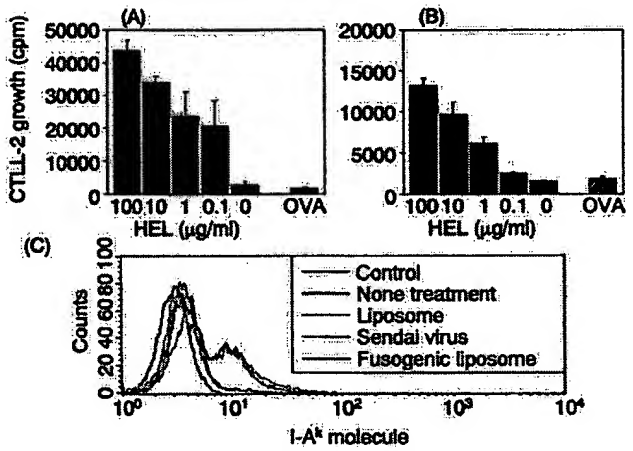

FIGURE 3. Fusogenic liposomes induced MHC class II-mediated Ag presentation of HEL. Macrophages (*A*) or IFN-γ pretreated MODE-K cells (*B*) were cultured with fusogenic liposomes containing HEL (■) or OVA (100 μg/ml, ▨) for 5 h, then Ag presentation was analyzed as described in *Materials and Methods*. Values are expressed as means ± SD of triplicate cultures. Fusogenic liposomes activated expression of MHC class II molecules on epithelial cells (*C*). MODE-K cells were cultured with conventional liposomes (pink), fusogenic liposomes (red), or Sendai virus (light blue). Cells were stained with anti-I-A^k Ab 48 h later. Expression was determined using a FACScan flow cytometer. Data are representative of three separate experiments.

Induction of OVA-specific CTL responses

Ag-specific Th1-type responses in addition to Th2-type responses were induced by nasal immunization with OVA-fusogenic liposomes (Fig. 5). Thus, we examined whether or not fusogenic liposomes can induce MHC class I-mediated OVA-specific CTL responses. CTL activity against EG7 was detected in the spleens of mice immunized with OVA-fusogenic liposomes following in vitro restimulation with Ag (Fig. 8*C*). In contrast, spleens isolated from mice immunized nasally with OVA alone or OVA-liposomes did not show OVA-specific CTL activity (Fig. 8, *A* and *B*). In addition, mononuclear cells from CLN and MLN of mice nasally immunized with OVA-fusogenic liposomes also showed OVA-specific CTL activity after in vitro restimulation with Ag (Fig. 8*C*). Furthermore, high levels of the mRNA for perforin that is a major cytotoxic molecule of CTL were expressed in the nasal passages of mice nasally immunized with OVA-fusogenic liposomes but not with OVA-liposomes or OVA alone (Fig. 8*D*). These findings suggested that nasally administered OVA-fusogenic liposomes induced Ag-specific CTL responses.

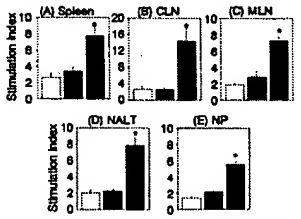

FIGURE 4. Nasally administered OVA-fusogenic liposomes induced Ag-specific proliferative responses of $CD4^+$ T cell. Seven days after the final nasal immunization with various forms of OVA (□, OVA alone; ▨, OVA-liposomes; ■, OVA-fusogenic liposomes), $CD4^+$ T cells from spleen, CLN, NALT, nasal passages (NP), and MLN were isolated and cultured with 1 mg/ml OVA. Proliferative responses were determined by [^{3}H]thymidine. Error bars indicate means ± SE for four mice analyzed separately in triplicate assays. *, $p < 0.01$ vs OVA-liposomes.

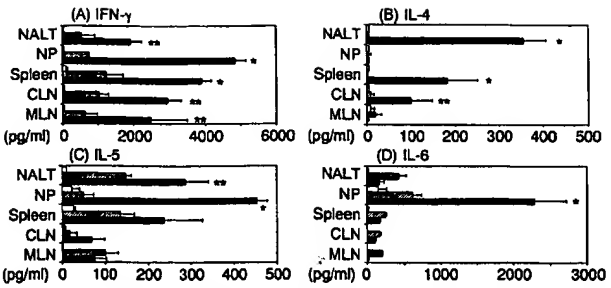

FIGURE 5. Characterization of Th1 (IFN-γ)- and Th2 (IL-4, IL-5, and IL-6)-type cytokine productions by OVA-specific $CD4^+$ T cells. Seven days after the final nasal immunization, $CD4^+$ T cells from spleen, CLN, NALT, nasal passages (NP), and MLN were isolated and cultured with 1 mg/ml OVA for 96 h. Cytokine productions in the culture supernatants were determined by the appropriate cytokine-specific ELISA (□, OVA alone; ▨, OVA-liposomes; ■, OVA-fusogenic liposomes). Error bars indicate means ± SE for four mice analyzed separately in triplicate assays. * and **, $p < 0.01$ and $p < 0.05$, respectively (vs OVA-liposomes).

Discussion

It is important to develop novel strategies to induce Ag-specific immune responses at both mucosal and systemic compartments for the prevention of infectious diseases localized at or introduced by mucosal surface (1–3). In this study, we demonstrated the potential of fusogenic liposomes as a nasal vaccine vehicle. We previously reported that fusogenic liposomes were composed of conventional liposomes and Sendai virus and attached to the cell membrane using envelope glycoproteins of Sendai virus (11, 13–15). In addition, it is well known that Sendai virus naturally infects via mucosal epithelia (16, 17). In this context, in vivo Ag distribution assay showed that fusogenic liposomes also attached to the nasal epithelia and delivered the encapsulated Ag (Fig. 1, *A* and *C*), according to the high Ag-delivery activity to mucosal epithelial cells in vitro (Fig. 1*G*). In contrast, conventional liposomes as well as Ag alone could not deliver the Ag into the nasal immune systems. It has been reported that the conventional liposome is a good candidate as an Ag delivery vehicle, especially for s.c. immunization (27, 35, 36). However, in the case of mucosal vaccine, the conventional liposome is not effective because mucosal barriers prevent the incursion of the conventional liposome into the nasal immune system.

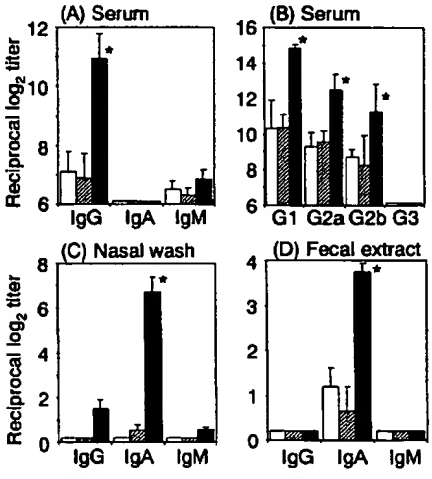

FIGURE 6. OVA-specific Ab responses after nasal immunization with OVA-fusogenic liposomes. Serum IgG, IgA, and IgM responses (*A*), serum IgG subclass responses (*B*), IgG, IgA, and IgM responses in nasal washes (*C*), and in fecal extracts (*D*) were determined by ELISA (□, OVA alone; ▨, OVA-liposomes; ■, OVA-fusogenic liposomes). Findings are expressed as means ± SE of four mice analyzed separately in triplicate assays. *, $p < 0.05$ vs OVA-liposomes.

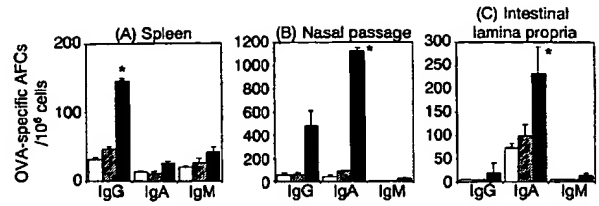

FIGURE 7. Induction of Ag-specific AFCs in mucosal and systemic compartments of mice nasally immunized with OVA-fusogenic liposomes. OVA-specific IgM, IgG, and IgA AFCs in spleen (*A*), nasal passages (*B*), and intestinal lamina propria (*C*) were determined by ELISPOT assay (□, OVA alone; ▨, OVA-liposomes; ■, OVA-fusogenic liposomes). Findings are expressed as means ± SE of four mice analyzed separately in triplicate assays. *, $p < 0.05$ vs OVA-liposomes.

It has been reported that M cells are morphologically different from neighboring epithelial cells and are specialized for the uptake and transcellular transport of particle Ags and microorganisms from the lumen to the lymphoid follicles (30–32). Thus, it seems that M cell is a target cell for the efficient delivery of vaccine Ag into NALT. In this regard, we examined whether or not fusogenic liposomes fused and delivered the Ag to M cells. Confocal microscopic analysis using M cell-specific lectin revealed that fusogenic liposomes delivered the Ag to M cells as well as to neighboring epithelial cells (Fig. 2). These findings demonstrated that fusogenic liposomes constitute an effective Ag delivery system for M cells, epithelial cells, and $Mac\text{-}1^+$ cells in nasal immune compartments.

In this study, it was also demonstrated that Ag delivered by fusogenic liposomes were presented with MHC class II molecules of epithelial cells as well as macrophages (Fig. 3, *A* and *B*). Several studies concerning the Ag presentation ability of mucosal epithelial cells have been reported (37–39). These studies showed that epithelial cells had the potential to present Ags via both MHC class

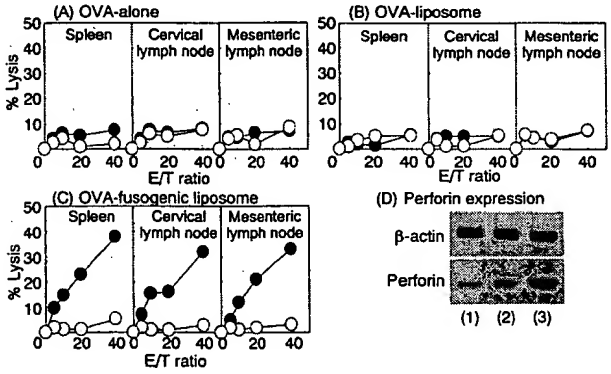

FIGURE 8. Nasal immunization with OVA-fusogenic liposomes induced OVA-specific CTL responses. Seven days after the final nasal immunization, mononuclear cells from the spleen, CLN, and MLN of mice nasally immunized with OVA alone (*A*), OVA-liposomes (*B*), or OVA-fusogenic liposomes (*C*) were isolated and restimulated with MMC-treated EG7 for 5 days to enhance the frequency of Ag-specific CTLs. CTL activity against EG7 (●) or EL4 (○) were measured by ^{51}Cr release assay. Messenger RNA expression of perforin in nasal passages was determined (*D*) as described in *Materials and Methods* (*lane 1*, OVA alone; *lane 2*, OVA-liposomes; *lane 3*, OVA-fusogenic liposomes). Each analysis was performed at least three times.

I and II molecules and to activate T cells with costimulatory molecules. It has been also shown that several viruses stimulated the expression of MHC molecules on infected epithelial cells (33, 40). In this respect, we found that fusogenic liposomes are also capable of inducing epithelial cells to express MHC class II molecules (Fig. 3*C*). These data suggest that fusogenic liposomes had an adjuvant activity against epithelial cells to enhance the MHC class II-mediated Ag presentation.

As shown in Figs. 4 and 5, fusogenic liposomes induced high levels of Th1 and Th2 responses. It is interesting to note that two distinct patterns of Th2-type cytokines were induced between inductive (NALT) and effective (nasal passage) sites of the nasal immune system. In the NALT of mice immunized with OVA-fusogenic liposomes, high levels of IL-4 produced by Ag-specific $CD4^+$ Th2 cells may provide a molecular environment for the preferential induction of Ig class switching from μ to α H chains. IL-4 supports TGF-β-induced IgA-specific class switching in NALT (41–43). Additionally, another group of Th2-type cytokines including IL-5 and IL-6 induces the differentiation of IgA-committed B cells to plasma cells in the mucosal effector site (41–43). Thus, high production of IL-5 and IL-6 was observed in $CD4^+$ T cells isolated from the nasal passages of mice nasally immunized with OVA-fusogenic liposomes. Furthermore, the induction of Th1-type cytokines such as IFN-γ indicates the simultaneous generation of cell-mediated immunity including CTL. In addition, the production of IFN-γ, especially by nasal passage $CD4^+$ T cells, may create an optimal molecular environment for the efficient production of Ag-specific secretory IgA synthesis, because the induction of the secretory component (or poly-Ig receptor) is up-regulated by IFN-γ (44).

As expected based on the cytokine profile of Ag-specific Th1 and Th2 cells, it was shown that fusogenic liposomes induced high levels of OVA-specific mucosal and systemic Ab responses (Figs. 6 and 7). According to previous studies, the coadministration of mucosal adjuvant was essential to generate Ag-specific mucosal and systemic immune responses via the respiratory and gastrointestinal immune system (6, 8). A separate study showed that nasal vaccination with *Streptococcus pneumonia* pneumococcal surface protein A and the nontoxic mutant cholera toxin S61F induced protective immunity through Ag-specific mucosal IgA and systemic IgG Ab responses (7). However, nasal vaccine containing pneumococcal surface protein A alone did not cause the generation of Ag-specific Th and B cell responses. In contrast, fusogenic liposomes can effectively induce Ag-specific Th1 and Th2 cells in addition to the associated IgG and IgA Ab responses in both mucosal and systemic sites without mucosal adjuvant. Additionally, OVA-specific CTL responses were induced following nasal immunization with OVA-fusogenic liposomes (Fig. 8). Direct intracellular delivery of Ag via the fusion process may guide an encapsulated Ag to the MHC class I pathway. In support of this, we have already shown that fusogenic liposomes can deliver encapsulated Ags into the MHC class I-dependent pathway (14). These data strongly suggest that the fusogenic liposome is an effective nasal Ag delivery vehicle, especially against to virus infection due to their activities to induce Ag-specific CTL responses as well as Ab productions.

A similar vehicle (known as proteoliposome) was previously developed by the other groups (45–47). This proteoliposome has been prepared by reconstituting biologically active Sendai virus glycoprotein into conventional liposomes using a dialysis method (45–47). It was shown that rhesus monkeys develop Ag-specific CTL responses at the systemic compartments following systemic immunization with proteoliposomes containing SIV Ag (45). However, the application of the proteoliposome as a vaccine was somehow targeted only the induction of systemic immune response. Therefore, the present study is the first to demonstrate the feasibility of fusogenic liposomes as an effective mucosal Ag delivery system for the induction of mucosal as well as systemic immune responses.

In summary, this study demonstrated that novel hybrid fusogenic liposomes constitute a powerful mucosal vaccine delivery system that can elicit Ag-specific CTL, Th1/Th2, and IgG and IgA Ab responses in mucosal and systemic sites.

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Growth inhibition of human leukemia HL-60 cells by an antisense phosphodiester oligonucleotide encapsulated into fusogenic liposomes.

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We report here the antisense effect of phosphodiester oligodeoxynucleotide (D-ODN) using fusogenic liposomes (FL) as its carrier. FL has envelope proteins of the Sendai virus within its membrane and introduces its contents directly and efficiently into cytosol by means of the virus-cell fusion mechanism. Using antisense (AS) D-ODN 15-mer complementary to the c-myc proto-oncogene mRNA, including the translation initiation codon site, we analyzed the growth of HL-60 cells by [3H]-thymidine uptake. AS-ODNs encapsulated in FL inhibited the growth by about 70% that of the control HL-60 cells at 2.48 microM. In contrast, sense and scramble D-ODNs encapsulated in FL showed no effect of the growth of HL-60 cells at the same concentration. Even at 50 microM, free form D-ODNs did not show any effect. These results suggest that FL is potentially a useful delivery vehicle for oligonucleotide-based therapeutics, and that D-ODN may be a likely candidate for oligodeoxynucleotides when an efficient delivery system is used.

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